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Abstract

Telomeres are the repetitive sequences located at the ends of linear chromosomes, which protect the DNA from degradation during cellular replication. These structures are elongated by a ribonucleoprotein called telomerase. The protein component of telomerase (TERT) utilizes an RNA template sequence (TR) to reverse transcribe nucleotides onto the 3' end of the chromosome. While the TERT is highly conserved between organisms, the TR varies greatly in primary sequence and length, thus providing difficulties in identifying the TR of various organisms. We currently know only 3 groups of TRs from ciliates, humans, and yeast. I am interested in identifying the TR sequence in a filamentous fungus, Aspergillus oryzae, which I hypothesize uses a telomerase-based system to maintain the telomeres. Genomic searches for a sequence complementary to the 12 bp telomeric sequence produced 24 candidate TR sequences. I tested 5 sequences for its presence in the RNA by method of RT-PCR. Four of these sequences either did not produced a band for an *A. oryzae* RNA sample or for a DNA sample. However, 1 sequence did amplify an RNA segment, which indicates that it is transcribed. Additionally, this candidate template sequence follows the other criteria I determined important for the TR sequence. The identification of this filamentous fungus' telomerase RNA sequence can be further utilized in understanding the telomere length regulation.

Introduction

In 1948, a student by the name of James Watson set out on a mission to discover the structure of DNA. In an era where little was known about DNA, he had to combine all the little bits of information, some of which may have seemed unrelated, to find its structure. However, this finding would have one of the greatest impacts on current day molecular biology. Scientific research progresses into the unknown, where the right questions must be asked and the correct tests performed in order to gain insight on some biological phenomenon. Similar to Watson, I searched for the structure of another piece of genetic information, a specific RNA molecule. Despite the fact that I have technological advancements, such as whole genome sequences and the Internet, the same spirit of searching into the unknown remains. Little is known about the structure of this RNA, a component in an enzyme important for maintaining the ends of linear chromosomes. However, by examining the small details of what is known, my aim is to identify the RNA structure of telomerase in a filamentous fungus called Aspergillus oryzae. First, we must understand why this enzyme is important before explaining my steps towards identifying this sequence.

*This author wrote the paper as a senior thesis under the direction of Dr. Karen Kirk.

Structure of DNA

DNA (deoxyribonucleic acid) contains all the genetic information for a cell. This is composed of a deoxyribose-phosphate 'backbone' with 4 bases branching off from the sugar molecule (Watson and Crick, 1953). The 4 bases for DNA are adenosine (A), cytosine (C), guanine (G), and thymine (T). Because of the Watson-Crick base pairing (A with T and C with G), the two strands of DNA are held together by hydrogen bonding, which produces the typical alpha-helix structure.

The backbone of DNA is structured in a unique way that provides directionality for the strand. The 3rd and 5th carbons (termed 3' and 5') in the deoxyribose are the positions where the phosphates bind. Thus, all the 3' carbons are oriented towards one end of the DNA strand, while all the 5' carbons are oriented towards the other end, with phosphates bound between each sugar molecule. However, most enzymes that synthesize a nucleic acid strand, such as DNA polymerase, are only able to add free nucleotides onto the 3' position, which will have implications during cellular replication. Additionally, the two strands of the alpha-helix are anti-parallel. As Watson and Crick (1953) noted, the specific base pairing and directionality of the two strands provides a simple mechanism for replication, and indeed DNA is replicated in a semiconservative manner.

The End Replication Problem

When a cell divides to produce two daughter cells, the entire genome must be copied so that each cell receives a complete set of genetic blueprints. However, the proteins involved in this replication process cannot copy the complete genome. Therefore, cells that have linear chromosomes developed a region at the end, termed a telomere, which ensures the complete replication of the DNA. First, we need to understand the replication process in the absence of telomeres, which will then explain why telomeres are crucial for the survival of an organism.

If telomeres did not exist, the genes located near the ends of the chromosome would be lost to the daughter cells due to the repetitive inability to replicate the ends. As stated earlier, double-stranded DNA (dsDNA) is replicated in a semiconservative manner (Alberts et al., 1994, p. 102). This means a newly replicated dsDNA segment will contain a parental strand and a newly synthesized, complimentary strand. At the start of DNA replication, the 2 parental strands are separated. After this, DNA primase creates a short RNA segment on one strand, which serves as the starting point for synthesis (figure 1) (Alberts et al., 1994, p. 254-255; reviewed in Hug and Lingner, 2006). However, the DNA polymerase only functions in the 5' to 3' direction (Alberts et al., 1994, p. 253-254). Thus, when the two strands are separated, two different approaches must be made to completely synthesize the complimentary sequence.

Replication of one of the two strands of DNA involves constant activity of the DNA polymerase. This strand, termed the leading strand, starts with an RNA primer created away from the end, and the polymerase moves towards the 5' end of the parental DNA segment (figure 1, bottom strand) (Alberts et al, 1994, p. 252-253). The activity of the polymerase is uninterrupted and is able to reach the very last nucleotide in the strand. However, the other parental strand has another situation.

As mentioned previously, the polymerase only extends nucleotides in the 3' direction. The complementary, parental strand to the leading strand is directed in the opposite direction. Thus, this lagging strand has the 3' directed towards the end of the DNA strand (figure 1, top strand). To allow the DNA polymerase to move towards the 5' end of the parental strand, synthesis must start at the very end of the chromosome, where an RNA primer is created. Since the polymerase cannot start de novo, the RNA primer is critical to initiate replication. However, the RNA primer is degraded after replication starts, and there is no method to synthesize these missing nucleotides.

This loss of replication ability at the 3' ends on chromosomes can be critically important. If essential genes are located at the 3' end, they may be lost to daughter cells through the successive rounds of division. This detrimental effect would lead to the death of the organism. As a result, cells that contain linear chromosomes incorporated a system that accommodates some loss of DNA at the 3' end but does not allow the loss of important genetic information.

The Structure and Function of Telomeres

The structures at the end of linear chromosomes, called telomeres, are important in the cellular division process because they allow for complete replication of the genomic material. This region consists of a repetitive, non-coding dsDNA sequence, a G-rich, single-stranded (ssDNA) 3' overhang, and multiple binding proteins (figure 2) (reviewed in Ferreira et al., 2004). These repeat sequences are very short; they can vary from 5 nucleotides in silkworms and insects (Okazaki et al., 1993) and up to 25 nucleotides in the yeast Kluyveromyces lactis (McEachern and Blackburn, 1994). The first organism to have its telomeres sequenced was the ciliate Tetrahymena thermophila, which showed a repeat sequence of 5'-TTGGGG-3' (Blackburn and Gall, 1978). The sequence, 5'-TTAGGG-3', is found in numerous species including humans, mice, slime molds, and some fungal species, such as Neurospora crassa and Aspergillus nidulans (Kusumoto et al., 2003). On the other hand, some yeast species have a heterogeneous telomere repeat sequence, such as 5'-TTAC(A)(C)G1-8-3' for Schizosaccharomyces pombe and 5'-TG2-3(TG)1-6-3' for Saccharomyces cerevisiae (Duffy and Chambers, 1996).

Besides ensuring complete genomic replication, telomeres have a second role involved in identifying chromosome ends. Many proteins bind to the double- and single- stranded telomeric repeat sequence, such as TRF1, TRF2, and POT1 (reviewed in de Lange, 2005). These help make the core components (along with TIN2, Rap1, and TPP1) of a complex, called shelterin. Their role is to identify the telomeres as legitimate chromosome ends, rather than double stranded DNA breaks. Rap1 prevents the activation of the non-homologous end joining (NHEJ) pathway (Pardo and Marcand, 2005), where chromosome ends are fused together, while other proteins that associate with shelterin help prevent homologous recombination between sister chromatids at telomere ends, such as Ku (Celli et al., 2006). There are many other proteins involved in telomere maintenance, but their exact interactions remain unknown.

Telomeres in Aging and Cancer

On a cellular level, telomeres play an important role in the cell cycle. Telomeres have been connected with the aging of cells, by serving as a "biological clock," as well as being connected with cancer development. While these may seem very different, they are in fact related, as explained below.

In somatic cells, the telomeres become shorter with each cell division (Harley et al., 1990). When the telomeres reach a predetermined length, the cell enters a state of senescence, where it is metabolically active but no longer goes through cellular division (reviewed in Steward and Weinberg, 2006). Leonard Hayflick found in the 1960's that human fibroblast cells only replicated a defined number of times when grown in culture (reviewed in Stewart and Weinberg, 2006). This effect can be observed for most tissues in humans, such as skin cells. Additionally, the number of genetic mutations in a cell accumulates over time. Thus, the telomere shortening mechanism may act as a biological clock for the cell, signifying it to stop dividing to repress the chance of passing along defective genetic code.

To keep the cell in a senescent state, the cell must have activation of two tumor suppressor genes, p53 and Rb (reviewed in Stewart and Weinberg, 2006). However, mutations in both of these genes allow the cells to continue replicating, resulting in telomeres that become critically short. In this crisis stage, the chromosome ends fuse together (reviewed in Stewart and Weinberg, 2006) or the chromosomes rearrange and become increasingly unstable (reviewed in Kim et al., 2002), leading to a cell-lethal effect. However, in a subset of the cells that reach the crisis stage, further mutations in the DNA result in the maintenance of the telomeres. The telomere length increases to a stable size, allowing the cells to continuously divide unregulated (reviewed in Blackburn, 2001). If a somatic skin cell bypasses senescence and reaches the crisis stage due to numerous chromosomal mutations, but survives due to the activation of telomere maintenance, then the instability of the chromosome may result in immortal, cancerous cells, if certain conditions are present (reviewed in Kim et al., 2002; Stewart and Weinberg, 2006). Indeed, about 90% of all cancer cells have telomere maintenance activity, and there are currently many therapeutic agents that are being investigated to inhibit this maintenance system (Shay, 2005).

Along with cancerous cells, germ line cells must maintain a stable telomere length over time to allow the cellular divisions to continue. Thus, cells activate an enzyme called telomerase, which adds telomeric repeats to the 3' ends of chromosomes (Greider and Blackburn, 1985), increasing the telomere length. This enzyme has unique properties, and is critical for the regulation of telomere length.

Telomerase: Regulator of the Telomeres

As seen in somatic cells, telomeres undergo shortening after cellular division, even in cancerous and germ cells. However, the activation of an enzyme called telomerase in cancerous and germ cells maintains the longer telomeres when compared to the telomere length in somatic cells, which do not activate telomerase. This enzyme is a ribonucleoprotein that contains 2 essential elements: the reverse transcriptase enzyme and the RNA template sequence. The reverse transcriptase component, termed TERT (telomerase reverse transcriptase) or Est2p in yeast (Dandijnou et al. 2004), is the catalytic component of telomerase in that it adds nucleotides to the 3' end of the DNA. While all the telomeres shorten after cellular division, cancerous and germ cells use active telomerase to "reset" the telomere length to its original state.

The catalytic component of telomerase is very well conserved between eukaryotes (Nugent and Lundblad, 1998). Therefore, the variability in the telomere repeat sequences for different species depends on telomerase's second critical component: the TR (telomerase RNA) template. Telomerase uses this single-stranded RNA molecule as a template for the reverse transcription step. Thus, if the telomere repeat sequence is 5'-TTAGGG-3', then the RNA template must have the sequence 5'-CCCUAA-3'. Even beyond the template region, the TR

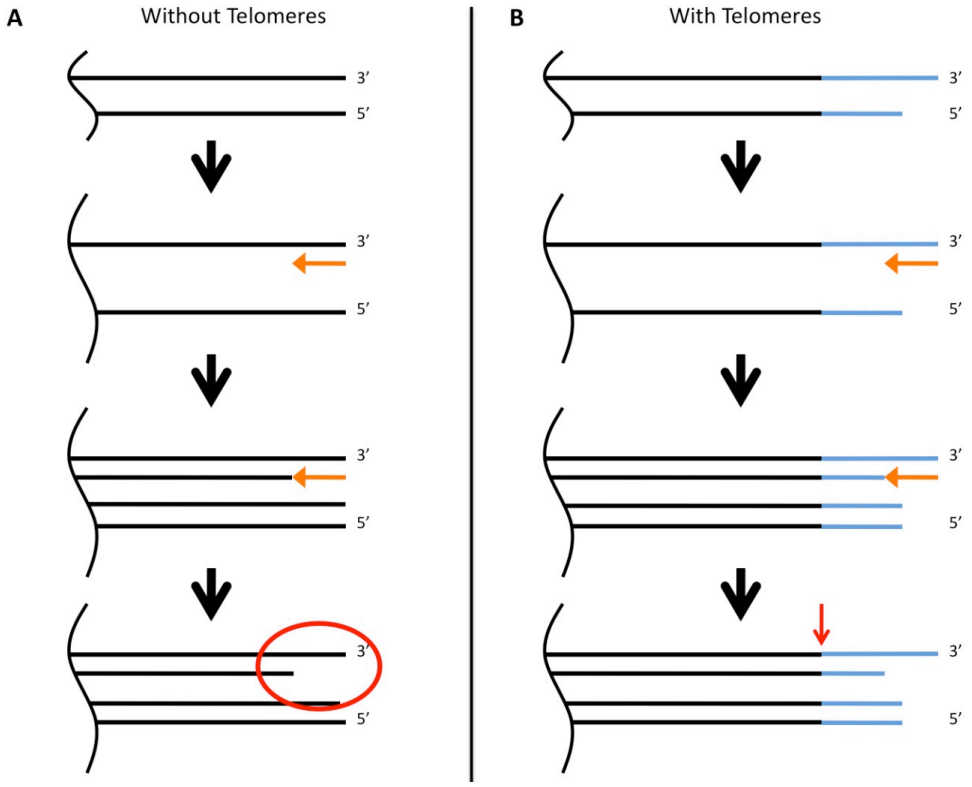

Figure 1. The end replication problem and the telomere solution. A) Schematic representing the end replication steps. First, the two strands are separated, and an RNA primer (orange) binds to the end of the lagging strand. After synthesis of the new strand, the primer is removed, leaving a lack of replication for the top strand. B) Same schematic as A, but with the presence of telomeres (blue). The primer will bind to the telomeric sequence, allowing for complete replication of both strands, as signified by the red arrow.

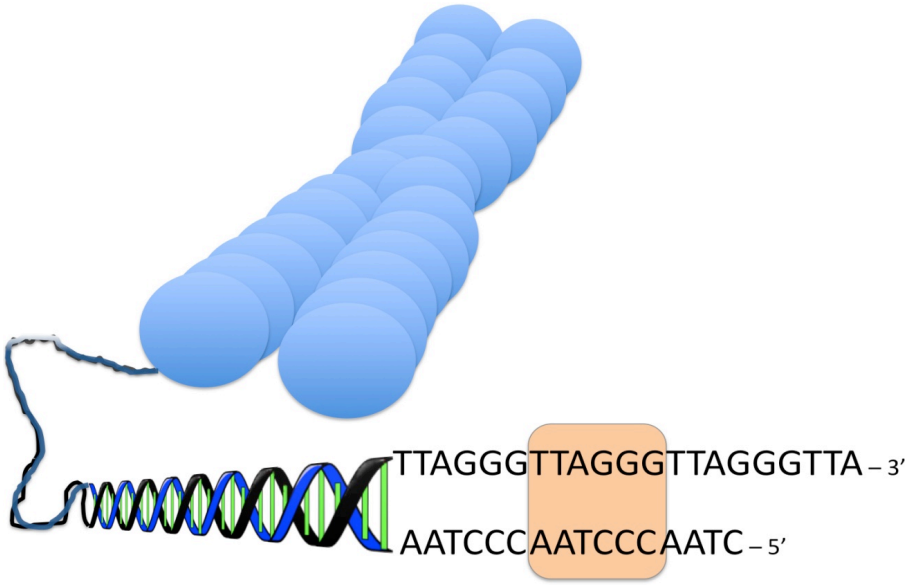

Figure 2. The telomere sequence at the end of a chromosome. A schematic of a condensed chromosome (blue) with the end sequence. One telomeric repeat of 5'-TTAGGG-3' is shown in an orange block.

components are very different, both in sequence length and nucleotide composition (Chen and Greider, 2004), and has not been identified in most organisms due to the lack of conservation.

Currently, we only know the TR sequences from three major groups of organisms: ciliates, vertebrates, and yeast (figure 3). The ciliate TR is only about 150 nucleotides in length (Greider and Blackburn, 1989), while the vertebrate TR vary between 400 and 550 nucleotides (Chen et al., 2000), and the yeast TR is around 1300 nucleotides in length (Dandjinou et al., 2004; Leonardi et al., 2008; Webb and Zakian, 2008). Even within the same class of organisms, differences exist. For example, in the vertebrate class, there are only 2 nucleotides between the template region and the 5' end for the TR in rodents, while there may be up to 50 nucleotides between these two regions in other vertebrates (Chen et al. 2000). This great variability in TRs has been one reason for the difficulty in identifying new sequences.

Despite the differences in sequence length, all TRs appear to fold into a similar secondary structure (figure 3) (Chen et al. 2000; Chen and Greider 2004). Just 5' to the template region, a boundary element exists which defines the template region. Additionally, this boundary element brings the template into a close proximity to a pseudoknot region, just 3' to the template, which has been suggested to facilitate binding to TERT (Chen and Greider 2004). Since TERT is fairly conserved between species, the proteins must contain a conserved TR binding domain, which would be observed in the TR structure. Indeed, in two yeast species, Saccharomyces and Kluyveromyces, the binding of TR (termed TLC1) to TERT (Est2p in yeast) depends mainly on the second stem in the pseudoknot region, which are similar in structure (Lin et al., 2004).

Surprisingly, not every organism uses a telomerase-based mechanism to regulate the telomere length. The telomeres in the fruit fly, Drosophila, are composed of long sequences that encode for retrotransposons (reviewed in Capkova Frydrychova et al., 2008), elements that produce transcripts and insert themselves into the genome. To elongate the telomere, these sequences amplify, and target the chromosome ends to synthesize a growing end. They can also use a recombination event between two chromosome ends, to elongate the telomere (reviewed in Capkova Frydrychova et al., 2008). However, this may be a unique situation of telomere maintenance, since most other organisms use telomerase to maintain telomeres.

Telomere Length Regulation

The main role of telomerase is to elongate the telomere, maintaining its original length. However, it remains unknown how cells determine this acceptable length and regulate it over many generations. It has been proposed that the cells use a "protein-counting" mechanism (Marcand et al., 1997), where long telomeres will be "measured" by having more protein bound to them and short telomeres will have less protein. However, this model of telomere length regulation cannot be applied across a wide range of species, since different species have different average telomere lengths. For example, the yeast Saccharomyces cerevisiae maintains an average telomere of 450 bp (Runge and Zakian, 1989), while humans maintain a telomere length of 10-14 kbp in the germ line cells (de Lange et al., 1990). Thus, each species must set its own acceptable telomere length, which must be regulated by a separate mechanism. Our lab is interested in understanding the telomere length regulation in a unique organism, which is explained below.

Aspergillus as a Model Organism

The Aspergillus genus contains over 185 species of filamentous fungi (Galagan et al. 2005). The most well known species is Aspergillus nidulans due to its role as an excellent model organism. Over 50 years of research on A. nidulans has increased our knowledge of various eukaryotic cellular properties, including metabolic regulation, development, cell cycle control, chromatin structure, cytoskeletal function, and DNA repair (Galagan et al. 2005; Todd et al., 2007). In 1997, the A. nidulans telomeric repeat sequence was found to be 5'-TTAGGG-3' (Bhattacharyya and Blackburn, 1997). Additionally, the overall length of the telomere was only about 15-20 repeats, or 90-120 nucleotides in length. This is quite surprising, because the human telomere repeat sequence is the same as in A. nidulans, but the length is about 100 times longer (de Lange et al., 1990). How this fungus maintains such short telomeres remains a mystery.

We currently have a protocol to quickly and easily assay the length of A. nidulans telomeres by PCR methods (Vahedi, 2008, personal communication), which can give us insights into the regulation of telomeres. If the telomere sequence in A. nidulans were mutated, we predict a disruption in length regulation resulting in shorter telomeres. One simple way to mutate the telomere sequence is to alter the telomerase RNA template sequence. Unfortunately, the RNA sequence is unknown in A. nidulans. We can predict the TR template sequence based on the telomeric repeat, but the remaining sequence cannot be determined. Since the TR is transcribed from DNA, a simple genomic search for the telomeric repeat sequence (5'-TTAGGG-3') would give potential gene locations. However, due to the six-nucleotide length, there are more than a thousand results, which make determining the correct sequence near impossible.

Fortunately, a related organism possesses a much longer telomere repeat sequence. Aspergillus oryzae maintains a twelve base telomeric repeat sequence of 5'-TTAGGGTCAACA-3' (Kusumoto et al. 2003). While *A. oryzae* is not a major research model organism, it is used widely in the production of fermented foods such as sake, miso, and soy sauce (Kusumoto et al. 2003). A previous study has shown that telomerase knockout experiments in Aspergillus resulted in shorter telomere lengths (Vahedi, 2008, personal communication), indicating that TERT is important for the regulation of the telomeres. Thus, I hypothesize that *A. oryzae* uses a telomerase-based system to maintain the telomeres. There is currently no known filamentous fungal TR sequence, and the identification of this sequence will allow further insight into the conserved structures that are common with the other known TRs.

Telomerase RNA Candidate Sequences in A. oryzae

The goal of my project is to identify the telomerase RNA sequence in *A. oryzae*. To achieve this, genomic searches for the telomeric repeat sequence will be utilized by the BLAST function, used on the National Institute of Technology and Evaluation (NITE) site, to identify candidate TR sequences. By using certain characteristics of the template, such as terminal redundancy (the extra nucleotides that aid the template in binding to the correct 3' position on the chromosome (Autexier and Greider, 1994)), chromosome location, and conservation in a related organism, I will be able to rank the candidates in order of favorability. Of the noted characteristics, the terminal redundancy is the most important, and I hypothesize that the template will have a high terminal redundancy due to the long template region. Indeed, the terminal redundancy for Tetrahymena contains 50% of the template sequence (6 bp) (Greider and Blackburn, 1989), while the terminal

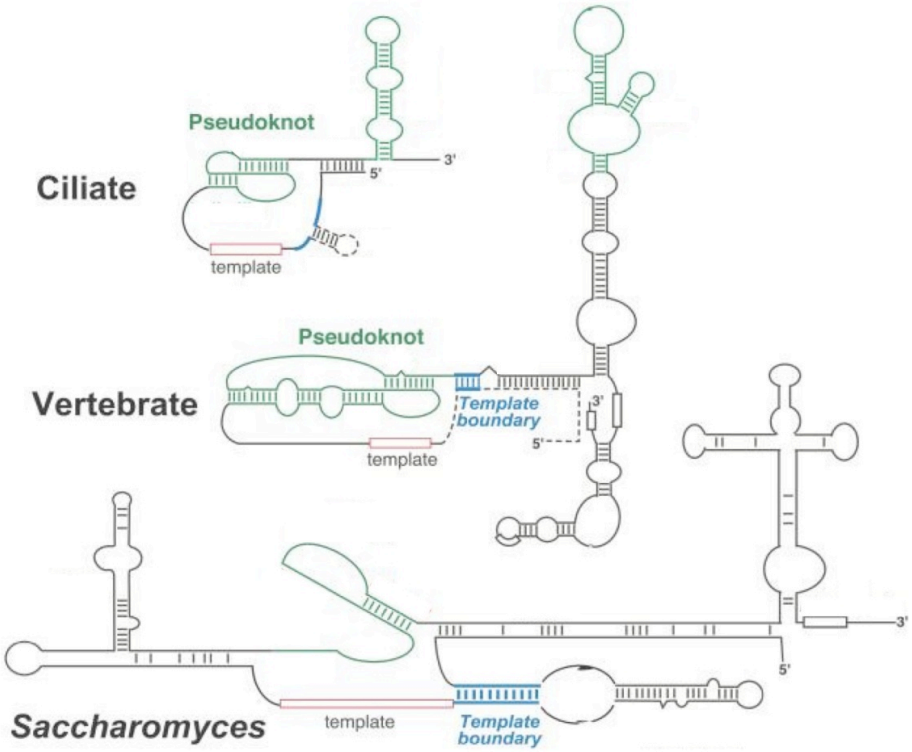

Figure 3. Telomerase RNA secondary structures. The three TR structures from ciliates, vertebrates and a yeast species, Saccharomyces, are shown. The ciliate sequence is ~150 bp, the vertebrate sequence is ~400-500 bp, while the yeast sequence is ~1300 bp. The common structures between all three are the single-stranded template, a template boundary element just 5' to the template, and close proximity of the pseudoknot to the template. Figure modified from Chen and Greider, 2004.

redundancy for humans contains 83% of the template sequence (6 bp) (Feng et al., 2005).

Since the TR must be transcribed from the genomic material, I need a test that can indicate whether a sequence is present in both the DNA and the RNA population. Thus, I will design 2 sets of primers that can amplify a portion of the DNA sequence surrounding the candidate template (a 5' and a 3' region). After polymerase chain reaction (PCR), two distinct bands sizes should be observed (figure 4A). To detect RNA, the same primers will be used for reverse transcription-polymerase chair reaction (RT-PCR). If the template sequence is located closer to one end, such as the 5' end as shown in figure 4B, then amplification will only occur for one of the primer pairs. This will help to determine which of the candidate sequences are found in the RNA population. Some of the genomic search results may include sequences that are intergenic (regions between genes that do not encode for anything) and thus will not be transcribed, while other results may include proteins that, by chance, contain the same sequence as the potential TR template, which should be transcribed. However, only one of the results will contain the actual TR template sequence.

Identifying the TR gene in *A. oryzae* will require examining the perceived insignificant details of the candidate sequences, as well as differences in transcription of these sequences. By putting all the pieces together, much like Watson did, the correct TR sequence may be identified in *A. oryzae*, resulting in a wide impact on the molecular biology field. First, we can start to understand the ways in which the filamentous fungi maintain very short telomeres by using mutational analysis. Secondly, we can gain an insight into the evolution of this gene by comparison to the other known telomerase RNA sequences.

Results

The goal of this project was to identify the telomerase RNA sequence for *A. oryzae*. Only 3 groups of organisms have a known TR (vertebrates, ciliates, and yeast). Other sequences remain unknown because synteny searches have been unsuccessful due to variations in the primary sequences and the total lengths of the TRs. However, *A. oryzae* maintains a long telomeric repeat sequence, which makes such a sequence less likely to occur randomly in the genome. To reach the goal of identifying the TR, RT-PCR was utilized to determine if the selected candidate sequences were transcribed. This provided a relatively simple and cheap method to identify the presence of a sequence within the RNA population. However, not all sequences could be tested at once, so a ranking of favorable versus unfavorable sequences needed to be made.

Telomerase RNA Candidate Sequences

Since the telomere repeat sequence is known for *A. oryzae* (Kusumoto et al., 2003), the entire genome was searched for this sequence (5'-TTAGGGTCAACA-3'). The search program, BLAST, used on the NITE site produced many results. However, I only noted results that were greater than one repeat, but less than two, due to the terminal redundancy. The terminal redundancy indicates the number of repetitive nucleotides added to the template region, which aids the template to bind in the correct location on the 3' overhang, as shown with the Tetrahymena (Autexier and Greider, 1994) and the K. lactis TRs (Fulton and Blackburn, 1998). Any result that was within 100 bp from the chromosome end was discarded, as this was indicative of a telomere sequence. A total of 24 candidate sequences

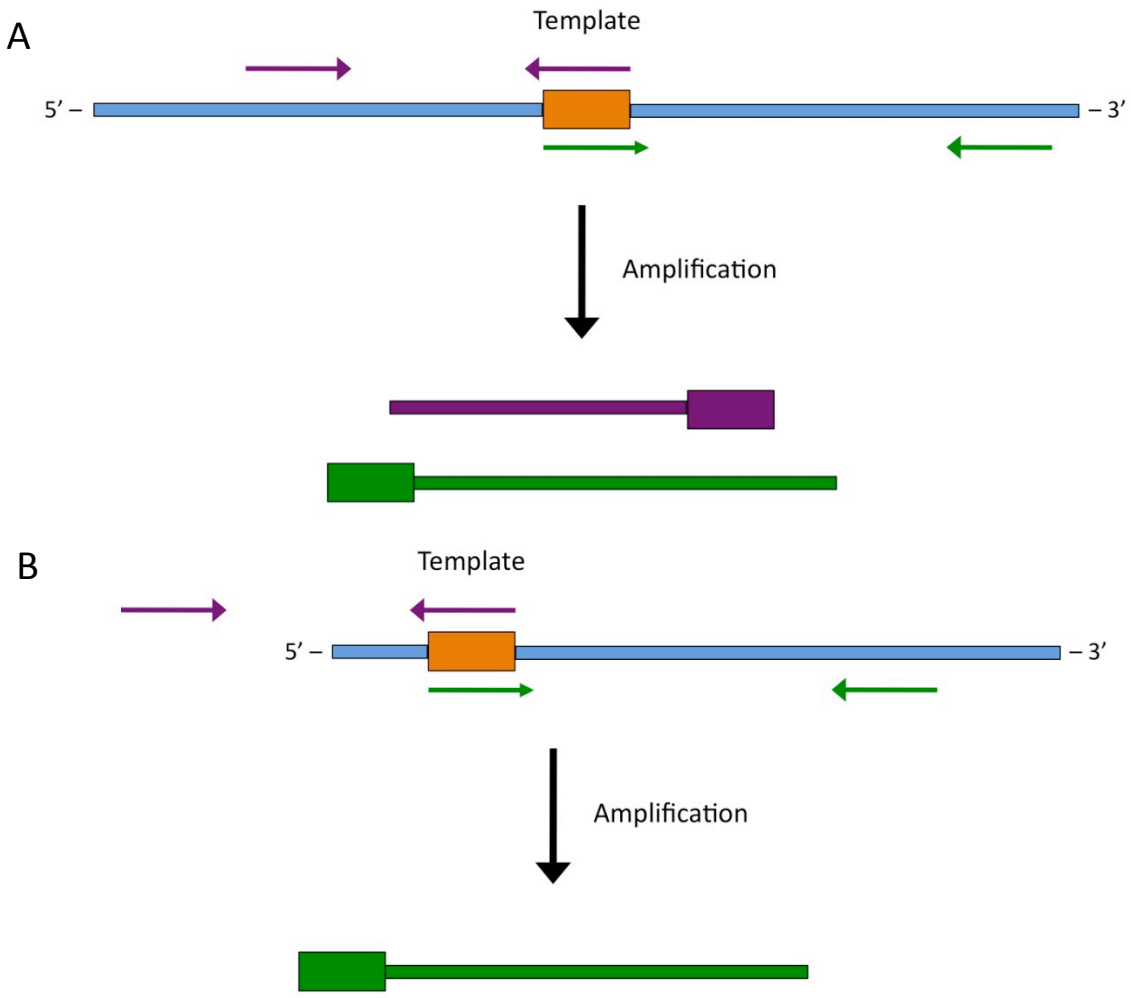

Figure 4. Schematic of products for PCR and RT-PCR. A) A hypothetical DNA sequence (blue) with the TR template (orange). Two different primer sets (purple and green) can amplify both the 5' and 3' region around the template after PCR. B) A hypothetical RNA sequence (blue) with the TR template (orange). Only the 3' region will by amplified, since the outermost primer on the 5' end does not bind.

remained, and each was recorded for its chromosome number and location (Table 1) (Meltser, 2007, personal communication). By noting the size of the chromosome, the location of the template region could be determined to be within 20 kbp from the terminal end or not. Another determining factor was the conservation of the sequence in a related organism, Aspergillus flavus. Since the telomere repeat sequence is conserved between *A. oryzae* and *A. flavus*, it is possible that the two TR sequences will share some identity. Thus, the immediate 200 bp region surrounding the possible template sequences was searched in *A. flavus* and were found to be either 100% identical (n=18) or minimally similar (n=6) (Table 1).

Based on the sequence characteristics in Table 1, I was able to selectively rank the sequences from unlikely to favorable. A sequence was unlikely to be the TR if the terminal redundancy was low (less than 3 nucleotides). Since the template region is relatively long, a greater terminal redundancy may be favorable. Furthermore, it seems unlikely to have the TR template sequence be exactly the same in two closely related species, *A. oryzae* and *A. flavus*, without also conserving some of the surrounding sequence. Finally, while the location of the sequence is not as important as the other three requirements, a result within an arbitrary 20 kbp from the terminus may be a false positive. For example, short, internal telomeric sequences in Chinese hamster cells were the proposed result of chromosome breakage points (Faravelli et al., 2002). Due to these characteristics, some unfavorable sequences include, but are not limited to, sequences 17 to 20 and J through T (Table 1).

Contrary to the unlikely sequences, the favorable sequences have the opposite characteristics. These have relatively longer terminal redundancies, are conserved in *A. flavus*, and are not located within 20 kb from the terminus. These favorable sequences include sequence A through I. However, in this study, I focused on sequences A, B, D, E, and H. Because of the ranking of sequences, I was able to examine the transcription of the favorable sequences before the unfavorable ones by RT-PCR on total, cellular RNA.

RT-PCR Set-up

Before I could determine if the possible telomerase RNA sequence is transcribed, a control was needed to indicate that detection of an RNA molecule is possible. Since telomerase contains both RNA and protein components, both need to be transcribed. The telomerase protein makes

Table 1. Possible telomerase RNA template sequences, location, and characteristics.

Label	Sequence	Chromosome	Contig	Location	Terminal Redundancy	Within 20kb from terminus?	Conserved in *A. flavus*
17	ATTAGGGTCAACA	4	SC102	1323888-1323900	1	No	Yes
18	CATTAGGGTCAACATT	7	SC206	266501-266490	4	No	No
19	ATTAGGGTCAACAT	1	SC308	30951-30963	2	Yes	No
20	TTAGGGTCAACAT	1	SC005	2402173-2402185	1	No	Yes
A	TTAGGGTCAACATTA	6	SC138	7140-7154	3	Yes	Yes
B	TTAGGGTCAACATTA	2	SC001	14311-14297	3	Yes	Yes
C	GGTCAACATTAGGGTCAAC	3	SC023	706319-796301	7	No	No
D	AGGGTCAACATTAGGGT	8	SC010	12593-12577	5	Yes	Yes
E	CATTAGGGTCAACATTA	8	SC103	1210918-1210934	5	No	Yes
F	ATTAGGGTCAACATTA	1	SC009	9156-9141	4	Yes	No
G	CATTAGGGTCAACATT	7	SC011	1779381-1779366	4	No	Yes
H	GGTCAACATTAGGGTCA	3	SC023	568952-568968	5	No	Yes
I	GGTCAACATTAGGGT	4	SC102	1152895-1152881	3	No	No
J	ATTAGGGTCAACAT	5	SC113	1730671-1730684	2	No	Yes
K	AACATTAGGGTCAA	4	SC012	1895138-1895151	2	No	No
L	GTCAACATTAGGGT	7	SC011	2109850-2109837	2	No	Yes
M	TAGGGTCAACATTA	8	SC010	660891-660878	2	No	Yes
N	GGGTCAACATTAG	6	SC138	452707-452695	1	No	Yes
O	TTAGGGTCAACAT	5	SC111	1595058-1595070	1	No	Yes
P	TTAGGGTCAACAT	3	SC026	1940846-1940858	1	No	Yes
Q	TTAGGGTCAACAT	2	SC003	1814888-1814876	1	No	Yes
R	AGGGTCAACATTA	8	SC010	405267-405279	1	No	Yes
S	TTAGGGTCAACAT	8	SC010	717630-717642	1	No	Yes
T	GTCAACATTAGGG	4	SC102	237446-237434	1	No	Yes

a good control because it should be actively transcribed throughout every cell (Masutomi et al., 2003).

In order to confirm the presence of the TERT sequence in the RNA population, RT-PCR was performed on isolated *A. oryzae* RNA. Additionally, PCR on *A. oryzae* DNA confirmed the usefulness of the primers. The expected size of the transcript was 653 bp (figure 5, lane 5), which matches the same size found when DNA was the template material (lane 6). I know this is pure RNA because when RNase If was added to the RNA sample, no bands appeared (lane 7). If there was contaminating DNA, it would have been amplified during the PCR cycles. The lack of such a band indicates that the band in lane 5 is dependant upon the presence of RNA. From this result, I concluded that the TERT primers could be used correctly on RNA and DNA to indicate a positive control for future experiments.

Despite the positive results with the TERT primers, the use of RNase If was the only method that indicated RNA was present in the reaction. Thus, switching to a different protein that goes through splicing, where introns are removed, could indicate the difference between RNA and DNA based on size differences. The ubiquitous protein, α-Tubulin, was chosen due to its many introns. Additionally, α-Tubulin is a structural protein that is expressed in every cell (McGrath et al., 1994). To determine if this sequence can be detected by both RT-PCR and PCR, primers were designed to different exons in *A. oryzae*, spanning 3 introns of a total length of 205 bp. Figure 5 shows that RT-PCR on RNA can detect both the primary transcrip and the spliced mRNA sequences. The clearly defined bands in lane 3 show the spliced portion of the mRNA sequence to be 267 bp, while the primary transcript portion to be 472 bp (205 bp longer due to the presence of the introns). As expected, the PCR results on the DNA show only one defined band at 472 bp (lane 4). Additionally, the addition of RNase If resulted in no band (lane 2). In conclusion, the use of RT-PCR could be used to test for transcription by using primers for 2 different sequences, TERT and α-Tubulin. However, the benefit of using the α-Tubulin primers is its ability to detect both sizes of the RNA sequence along with the single DNA size.

Since the positive and negative controls were working well, I decided to start testing the potential TR sequences for their presence in the RNA population. For each sequence, a total of 4 primers were designed (see figure 4). Two primers were targeted to the TR template sequence but in opposite directions, since this part must be included in the RNA sequence. Additionally, the smallest known TR sequence is only ~150 bp (Chen and Greider 2004), but the possibility for the *A. oryzae* sequence being around that length cannot be ruled out. Thus, the 2 other primers were targeted for a short region up- or down-stream of the template, about 100-150 bp away. Another benefit of this system is that it includes the possibility that the RNA template is at an extreme end of the TR sequence, as seen in the rodent TR (Chen et al. 2000).

Sequences B and E are Not Transcribed

For the 5' region to the candidate template for sequence B, I designed primers to amplify a region of 98 bp (termed B5). On the other side, the 3' region, I designed primers to amplify a region of 141 bp (termed B3). This nomenclature, of candidate template letter then region amplified, is used throughout this study.

Sequences B and E were favorable candidate TR sequences. Thus, to test if the B and E sequences were found in the RNA population, RT-PCR was performed on RNA. Similar to the primers for sequence B, primers for sequence E5 were designed to amplify a 157 bp region,

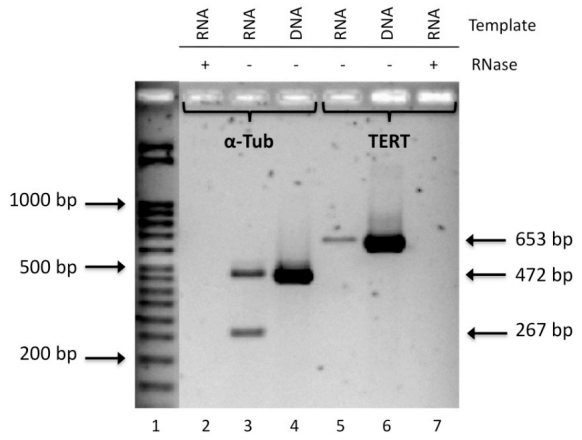

Figure 5. Transcription of α-Tubulin and TERT. RT-PCR was performed on an RNA starting template, while PCR was performed on a DNA starting template. The primers for α-Tubulin (sequence given in table 3) are able to amplify a portion of both the spliced mRNA (267 bp) and the primary transcript RNA (472 bp) (lane 3), while they are only able to amplify the longer sequence for the DNA (lane 4). The same size of 653 bp is amplified from RNA (lane 5) and DNA (lane 6) by use of the TERT primers. When RNase was added to the reaction, all RNA was degraded and no bands were produced (lanes 2 and 7), which serves as a control. Lane 1 is the DNA marker with sizes indicated.

while primers for sequence E3 were designed to amplify a 118 bp region. In figure 6A, the result of the test for sequence B transcription is shown. Firstly, the results are valid because of the expected positive and negative controls.

The α-Tub primers on RNA show the result of both the spliced and primary transcript correct sizes (lane 8), while the same primers on DNA only showed the larger size (lane 9). Additionally, when RNase was added to the RNA sample, no band was produced, as seen in lane 10.

The results for sequence B show that a band does not appear for either B5 or B3 RNA samples (figure 6A, lanes 2 and 5, respectively). However, the primers do work for the sequence since the PCR on the DNA sequences produced bands of the expected size (lanes 3 and 6). The faint band above the B3 DNA sample is unknown. Thus, I can conclude that sequence B is not transcribed.

Similar to sequence B, sequence E is not found in the RNA population of *A. oryzae*. Figure 6B was produced by the same methods as figure 6A, with the lanes corresponding to the same RT-PCR or PCR conditions, except for the primer sequences. As shown, no bands appeared for the RNA samples (lanes 2 and 5), but the DNA samples produced the correct size (lanes 3 and 6). Additionally, the controls worked as expected (lanes 8-10). In conclusion, both sequences B and E are not in the RNA population, which suggests neither were transcribed.

Sequences A and D: An Error in the DNA Code?

Since candidates A and D were favorable candidates, I tested these sequences for transcription using the same method as my previous sequences. However, using two different primer pairs produced unanticipated results.

On the original test for transcription, I designed primers for sequence A5 to amplify a 118 bp region, while primers for A3 were designed to amplify a 154 bp region. Additionally, primers for sequence D5 were designed to amplify a 148 bp region, while D3 primers were designed to amplify a 123 bp region. The results in a previous

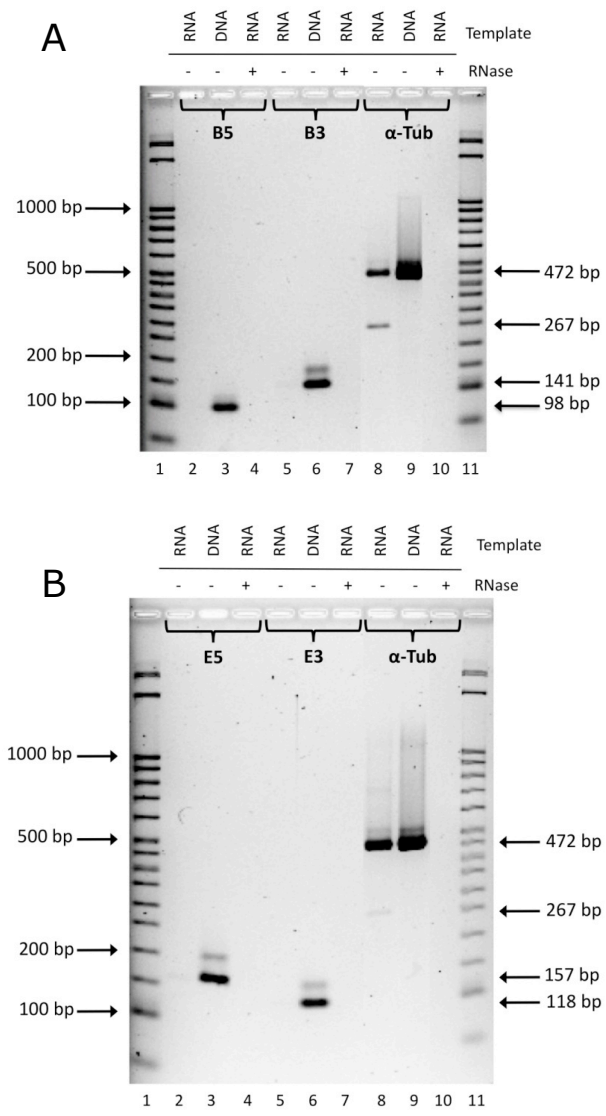

Figure 7. Amplification of sequences A and D on DNA do not work. Amplification by PCR methods on isolated *A. oryzae* DNA. A) Primer set 1 did not result in any visible bands for sequence A and D (lanes 2-5). However, the reaction worked, as shown with the expected size of the α-Tubulin band (lane 6), and the negative control (lane 7). Lane 1 is the DNA marker with indicated sizes. B) Primer set 2 also did not result in any visible bands for sequences A and D (lanes 2-5). The controls were as expected (lanes 6 and 7). Lane 1 is the DNA marker with indicated sizes.

experiment showed that the primers for sequence D produced no bands for either RNA or DNA (data not shown). Since all the other primers have amplified *A. oryzae* genomic DNA significantly, and the D primers were not designed differently than the rest, these should have produced at least a band for the DNA template. Because of this, I ran just PCR on DNA with the primers for sequences A and D (figure 7A). The reaction conditions were good, as shown with the visible α-Tub band at the expected size (lanes 6) and the lack of a band in the negative control sample (lane 7). However, no reaction produced any amplification of sequences A or D (lanes 2-5). The lack of any band led me to question the binding capabilities of the primers.

If the primers were not binding to the separated DNA strands, then no amplification could occur. Thus, I designed a second set of primers for sequences A and D. The targeted sequences were still kept close to the template region, but moved slightly 5' or 3' of the template. The primers for A5-2 (where "-2" indicates the second set for sequence A5) were designed to amplify a 121 bp region while the A3-2 primers were designed for a 165 bp region. Similarly, the primers for D5-2 were designed to amplify a 145 bp region while the D3-2 primers were designed for a 128 bp region.

Figure 7B shows the results from the PCR reaction on A. oryzae DNA with the second set of primers for sequences A and D. Again, the reaction did work, as shown with the positive results for the α-Tub control (lanes 6 and 7). However, the A and D sequence primers produced no bands (lanes 2-5). After using two different sets of primers for the same sequence, and the lack of any band appearing for any primer, leads me to suggest that there could still be an error in the primer binding capabilities , or that there may be an error in the genetic code, which would need to be investigated further.

Sequence H is Transcribed

Sequence H was another favorable candidate for the TR sequence. To test if the H sequence was found in the RNA population, RT-PCR was performed on RNA. Figure 8 shows that sequence H is found in the RNA population. This reaction was functional since the positive controls (lanes 6 and 7) and the negative controls (lanes 8 and 9) produced results as expected. For sequence H5, an equal sized band appears in lanes 2 and 3, despite the difference in starting template. Additionally, a faint band is present for H3 (lane 4), which also matches in size with that of the DNA (lane 5). Comparing the produced sizes with the DNA ladder (lanes 1 and 10) indicates these are of expected sizes. Thus, I could conclude that sequence H was found within the RNA population.

Summary: Which Sequences are Transcribed?

To identify the telomerase RNA sequence in A. oryzae, I first used an in silico method to identify candidate sequences. Out of the 24 total results, with various characteristics, a ranking system based on these characteristics was created. This led me to test sequences A, B, D, E, and H for their presence in the A. oryzae RNA population. Table 2 summarizes the RT-PCR on RNA results, along with the PCR on DNA results for all 5 sequences. While Sequences B and E produced the correct sized products for DNA, no bands were detected on the RNA samples. Unexpectedly, sequences A and D produced no bands for either the RNA or the DNA samples. Sequence H was the only one to produce the correct sized products for both the RNA and DNA samples.

Based on these results for presence in the RNA population, only sequence H is further supported as being the TR sequence for A. oryzae. I have shown that an RNA molecule exists in A. oryzae and that it contains a sequence complementary to the unique 12 bp telomeric repeat sequence.

Discussion

I hypothesized that Aspergillus oryzae utilizes a telomerase-based mechanism of telomeric DNA synthesis, and thus carries a gene that encodes a telomerase RNA in its genome. Therefore, the purpose this study was to narrow down the possible candidates for the telomerase RNA sequence in A. oryzae, based on their transcription. During

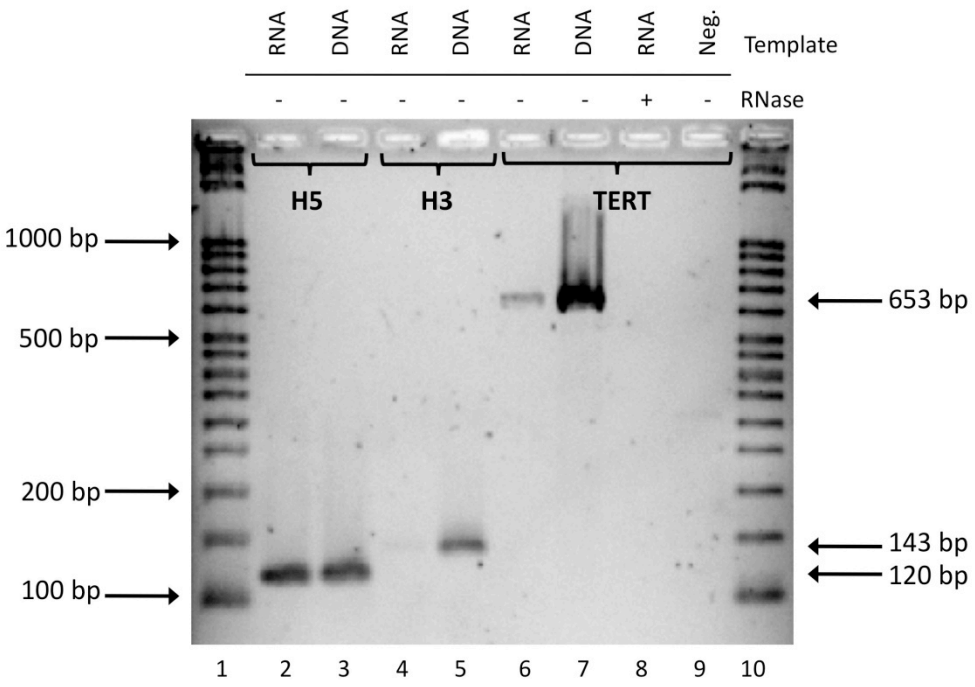

Figure 8. Sequence H is transcribed. RT-PCR was performed on an RNA starting template, while PCR was performed on a DNA starting template. Lanes 2 and 4 show the amplification of RNA for the indicated sequence, resulting in the expected size. This supports these sequences as being transcribed. The sizes of the RNA bands match with the sizes for the respective DNA (lanes 3 and 5). The controls produced expected results (lanes 6-9). Lanes 1 and 10 are the DNA marker with sizes indicated.

Table 2. Summary of candidate template transcription: Only sequence H is transcribed.

Genomic Material	Candidate Template Sequence: B	E	A	D	H
RNA	✗	✗	✗	✗	✓
DNA	✓	✓	✗	✗	✓

this process, I have discovered one candidate sequence that is transcribed and that conforms to the criteria I defined as being important.

List of Candidate Template Sequences in A. oryzae

As expected, some of the sequences are not transcribed even though the template characteristics seemed favorable, such as the terminal redundancy. Sequences B and E provide evidence for this situation; neither sequence produced any band for A. oryzae RNA. This suggests that neither sequence is found within the RNA sample, purified from actively growing hyphae. Unexpectedly, some sequences produced questionable results, such as sequences A and D. After designing two different sets of primers for the sequences provided by the NITE site, neither amplified DNA. This leads me to believe there could be an error in the sequence provided on the NITE site. This result highlights the importance of designing primers that can be used to detect both RNA for RT-PCR and DNA for PCR methods.

Sequence H: Is this the Telomerase RNA?

In this study, I have shown evidence that sequence H is transcribed, which is one component of support for this being the telomerase RNA sequence. This transcript contains a sequence that is complementary to the telomere repeat sequence of 5'– TTAGGGTCAACA –3', with 5 additional template nucleotides (terminal redundancy) added. While the remaining sequence around the template is unknown, we can determine certain characteristics about this sequence just from the template.

Of the recorded characteristics for the candidate sequences, the terminal redundancy was the most important. This sequence helps to position telomerase on

Table 3: Primer sequences used in this study.

Primer Name	Sequence	Sequence	% G/C Content	Tm (°C)
A5 Frwd.	GGGACAATCTACATCCATGAGG	22	50	62.7
A5 Temp. Rev.	TAATGTTGACCCTAAATGTGCAAG	24	38	59.4
A3 Temp. Frwd.	TAGGGTCAACATTACTGCTACTC	23	43	71
A3 Rev.	CTAGCATTGTCGCAGCTGCAC	21	57	64.5
A5-2 Frwd.	TGGCTTTTGCACCTCGACTCG	21	57	64.5
A5-2 Temp. Rev.	CCAACGGATACTGGGGAAAGG	21	57	64.5
A3-2 Temp. Frwd.	GGTGACGTTTTGATTGGATCCC	22	50	62.7
A3-2 Rev.	CTGGCAGTGTCTAAAAGTCTCC	22	50	62.7
B5 Frwd.	TCAGTACACAGGATTTTCTCCTG	23	43	71
B5 Temp. Rev.	TAATGTTGACCCTAAAGAAAATCTC	25	32	58
B3 Temp. Frwd.	CTTTAGGGTCAACATTATCACTTG	24	38	59.4
B3 Rev.	CAGAGACGCACGAAGCATCG	20	60	64.5
D5 Frwd.	CGTAACAGGATCTGGACATGC	21	52	62.6
D5 Temp. Rev.	ACCCTAATGTTGACCCTCATGC	22	50	62.7
D3 Temp. Frwd.	AGGGTCAACATTAGGGTGTTAC	22	45	60.8
D3 Rev.	GGACAGATACTCGCACATTTGG	22	50	62.7
D5-2 Frwd.	GGCCTGATCTAGCATAAAAGCG	22	50	62.7
D5-2 Temp. Rev.	GTATTCGCAAGTGCAAGAGATC	22	45	60.8
D3-2 Temp. Frwd.	CTCCGAACAGAGGTTAGTTGG	21	52	62.6
D3-2 Rev.	CACAGAAGACGGTCGCGACG	20	65	66.6
E5 Frwd.	CTACGATAGGCGGCAATGGG	20	60	64.5
E5 Temp. Rev.	TAATGTTGACCCTAATGAAGTGG	23	39	59.2
E3 Temp. Frwd.	CATTAGGGTCAACATTAAAAGGTG	24	38	59.4
E3 Rev.	GACTTGTGAAACCGGTTGGGG	21	57	64.5

H5 Frwd.	GACCGTCTTCAGTGCCTGTG	20	60	64.5
H5 Temp. Rev.	TGACCCTAATGTTGACCAAGTC	22	50	60.8
H3 Temp. Frwd.	GGTCAACATTAGGGTCAGATTC	22	45	60.8
H3 Rev.	CCAAGGTCTCCTCTTTGTCTG	21	52	62.6
Oryzae TERT Frwd.	ATATGGTACCTTGCGGCTCCTTC	23	52	64.6
Oryzae TERT Rev.	ACCAGATTATTGCGGACGTGCTC	23	52	64.6
A-Tubulin Frwd.	GTTGCCAGATCGCCAATTCTTG	22	50	62.7
A-Tubulin Nested. Rev.	TGTTCGAGGCATCCTCCTTGC	21	57	64.5

the correct 3' telomere overhang (Autexier and Greider, 1994). Since *A. oryzae* maintains a 12 bp telomere repeat sequence (Kusumoto et al. 2003), I predicted the RNA template would contain a longer terminal redundancy. Indeed, sequence H contains a terminal redundancy of 5 nucleotides, which indicates that 42% of the template nucleotides are repeated. This percentage falls near the range of terminal redundancies for other organisms. The human terminal redundancy contains 83% of the telomere repeat sequence (5 out of 6 nucleotides) (Feng et al., 2005) while the Tetrahymena terminal redundancy contains 50% of the telomere repeat sequence (3 out of 6 nucleotide) (Autexier and Greider, 1994). An exception to this trend is *K. lactis*, where the terminal redundancy contains only 20% of the telomere repeat sequence (5 out of 25 nucleotides) (Fulton and Blackburn, 1998). Even though the H sequence contains a relatively less redundancy to template percentage (42%), it will be interesting to determine if this is enough to function in *A. oryzae*, or if a longer redundancy is needed.

In addition to the terminal redundancy, other characteristics are indicative of a telomerase RNA. The template for sequence H is not found within 20 kbp from the terminus of chromosome 3. This reduces the chance that the chromosome end could have folded upon the chromosome and inserted part of a telomere sequence internally (Faravelli et al., 2002). Also, the surrounding 200 bp of sequence H are 100% conserved in the related organism, A. flavus. This indicates that sequence H may play an important role, since it is found within both organisms.

Telomerase RNA Biogenesis

Recently, it was discovered that different sizes of the TR sequence could exist in one organism (Box et al., 2008). When total RNA from Schizosaccharomyces pombe was run on a northern blot and then probed for the TER1 sequence (the telomerase RNA), a major band of about 1200 bp was indicated with less intense bands at about 1330 bp and 1400 bp (Leonardi et al., 2008; Webb and Zakian, 2008). This indicated that the longer bands were polyadenylated, which occurs after RNA processing. However, polyadenylation usually occurs after RNA strands are processed by the spliceosome, a set of proteins that remove introns from the primary RNA transcript (Alberts et al., 1994, p. 171-176). Indeed, a small intron was discovered at the 3' end of TER1, and when this region was spliced, to produce the two mRNA fragments, the mature 3' end of the telomerase RNA was created (Box et al., 2008). This is due to incomplete action by the spliceosome, because a fusion of the two mRNA fragments (the final step of splicing) results in non-functional telomerase RNA (Box et al., 2008). Whether sequence H for *A. oryzae* contains splice sites similar to those found in *S. pombe* remains unknown. If it does, this can shed light on the generation of the functional 3' end of the telomerase RNA.

Telomere Length Regulation in A. oryzae

On a more global scale, the short telomeres in *A. oryzae* provide interesting questions about its regulation mechanisms. The telomere length in both *A. oryzae* and *A. nidulans* are roughly equal despite the differences in the telomere repeat sequence. *A. nidulans* maintains a 6 bp telomeric repeat of 5'– TTAGGG –3' (Bhattacharyya and Blackburn, 1997). If the telomere of *A. oryzae* can be mutated to the *A. nidulans* sequence, can the telomere regulation stay the same? Additionally, since the shelterin complex binds to the telomere at each repeat (de Lange, 2005), the number of proteins bound should be less in *A. oryzae* than in *A. nidulans* due to the longer repeat sequence. Thus, it is possible that there is a mechanism

that recognizes the total length of the telomere, rather than the distinct "protein-counting" mechanism as previously suggested (Marcand et al., 1997).

Future Studies

There are many procedures that must be accomplished in order to fully identify the telomerase RNA sequence in *A. oryzae*. First, the remaining candidates must be tested for transcription by performing RT-PCR with primers specific to those sequences. This will help to further narrow down the possible candidates. Ideally, none of the remaining sequences will be transcribed.

One way to determine the function of the identified transcripts can be based on size. I hypothesize that the telomerase RNA sequence in *A. oryzae* will be similar in size to that of yeast (~1300 bp). To determine the total size of the transcripts, Northern blots must be utilized. In this method, total RNA is ran through a denaturing gel, which destroys secondary structures in the RNA. Additionally, this process separates the RNA based on size. The separated RNA is then transferred to a membrane and immobilized. A probe is created that will specifically bind to one sequence only, along with detectable signal (radiation for a radioactive probe or light for a chemiluminescence probe) to determine the size of the immobilized RNA. Chemiluminescent probes have already been created, and the use of these in Northern blots will help to determine the size of the H sequence transcript. If the total size of the H transcript is less than 1300 nucleotides, but greater than 150 nucleotide, then further support can be given for this sequence being the telomerase RNA in *A. oryzae*.

Determining the total size of the transcripts can help to narrow down the possible candidate TRs. However, this is not the only method that must be performed to indicate the correct TR sequence. Once the entire length of a candidate TR has been identified in *A. oryzae*, a functional analysis must be performed to demonstrate the correct function of telomerase. In this method, both the TERT enzyme and the TR are added in vitro with a telomeric primer (Greider and Blackburn, 1985), and final sequencing of the product can show if the probe has been extended or not with the telomeric sequence. The probe will only be elongated if a TR sequence is added, not with some mRNA sequence that happens to contain the TR template sequence.

Conclusion

In this study, I have identified one candidate telomerase RNA sequence, H, that is strongly transcribed. The template for this sequence also fits the favorable characteristics expected for a TR template. Sequence H has a 5 bp terminal redundancy sequence, is not found within 20 kbp from the terminal end of the chromosome, and the surrounding sequence is found in *A. flavus*. However, the total size of this transcript must first be identified, which can also provide evidence for its function if it is similar to the yeast size. The original work presented here has opened the door to indentifying a non-homologous, non-coding RNA sequence involved in telomere maintenance.

Methods & Materials

Organism and Growth Media

Aspergillus oryzae A815 (FGSC, Kansas City, MO) (McCluskey 2003) was used in this study. The fungus was grown on Polypepton Dextrin (PD) media (Polypeptone Peptone 1%, Dextrin 2%, KH2PO4 0.5%, MgSO4 0.05% Casein Hydrolysate 0.1%, NaNO3 0.1%, Agar 2%) (modified from Takahashi et al 2006) at 30°C.

After one week of growth, conidia were collected. Using a bent glass pipet, the lawn of fungus growth was rubbed vigorously with a 0.2% Tween 20 (Polyoxyethylene-sorbitan monolaurate, Sigma, St. Louis, MO) solution. The collected conidia were washed with nanopure H2O in an Eppendorf 5810 R centrifuge at 5000 rpm for 3 minutes. Conidial concentration was determined by counting on a hemocytometer (C. A. Hausser & Son, Philadelphia, PA).

Isolation of DNA and RNA

Approximately 3 x 107 conidia were incubated at 30°C in PD media at 120 rpm for about 18 h. A mortar and pestle was used with liquid nitrogen to freeze conidia and ground to a fine powder. *A. oryzae* A815 RNA was isolated by using a RNease Plant Mini Kit (Qiagen Sciences, Germantown, MD). Aliquots of RNA were stored at -80°C. Additionally, *A. oryzae* A815 DNA was isolated by using a DNeasy Plant Mini Kit (Qiagen, Germantwon, MD). The DNA was stored at -20°C.

Search for Potential Sequences and Primer Design

Since the sequence of the telomere repeat is known (TTAGGGTCAACA), I was able to search for the template region within the genome of *A. oryzae*. I used the genome sequence from the National Institute of Technology and Evaluation (NITE) (http://www.bio.nite.go.jp/dogan/MicroTop?GENOME_ID=ao). I used the BLAST (Basic Local Alignment Search Tool) search to find regions that included less than two complete telomeric repeats, since the telomerase RNA template includes terminal redundancy to aid in binding to the telomere. If a result was one or two exact telomeric repeats, it could be assumed a telomere section, and was ignored. Sequences were then labeled 17-20 and A to T, and the terminal redundancy noted. Searches on Pubmed indicated if sequences were located within hypothetical proteins or if they were conserved in *A. flavus*.

Since the size of the telomerase RNA sequence is unknown, special circumstances had to be considered. Primers were designed for a very small region around the potential template region (100-150 nucleotides). Additionally, the template region was used at one end of the amplified section, since the position of the template region in the RNA sequence is unknown. Because of this, four primers were designed for each sequence (Table 3); two primers amplified a region 5' to the template, and the other two primers amplified a region 3' to the template (see figure 4). All primers were designed to have a optimal melting temperature between 62°C and 64°C. Primers were ordered from Operon Biotechnologies Inc. (Huntsville, AL). Upon receiving, the indicated volume of nanopure water was added to achieve a 100 µM concentration. A portion of this underwent another dilution to achieve a concentration of 25 µM, and all primer solutions were stored at -20°C.

Reverse Transcription

The isolated RNA was used as the template material for a reverse transcription step to create cDNA (complimentary DNA) that can undergo amplification by PCR. 1 µl of RNA was added to a reaction containing four dNTPs (10 mM each) (New England BioLabs, Ipswich, MA), 2.5 µM of forward primer, and 0.01% DEPC-treated nanopure H2O to achieve a final volume of 8 µl. To ensure the presence of RNA and not DNA, 1 µl of water was replaced with 50 U of RNase If (New England BioLabs, Ipswich, MA). If DNA were present in the RNA sample, a band would appear after the PCR cycles. This reaction incubated at room temperature for 10 minutes before heating all samples at 75°C for 5 minutes in a Peltier Thermal Cycler (MJ Research) to denature any secondary structures. Following this, NE Buffer for M-MuLV Reverse Transcription, 1X, along with 200U of M-MuLV Reverse Transcriptase (New England BioLabs, Ipswich, MA) was added for a final volume of 10 µl. All samples were incubated at 42°C for one hour in the Thermal Cycler, then at 90°C for 10 minutes to inactivate the enzymes. Following this, the samples were stored at -20°C.

PCR Conditions

To amplify the targeted sequences, a standard polymerase chain reaction (PCR) was set up. A 40 µl reaction volume contained either 1 µl of the cDNA from reverse transcription, or 1 µl of the isolated *A. oryzae* DNA for the template, along with 1.25 µM primer solutions (both a forward and a reverse). Half of the volume included the JumpStart REDTaq ReadyMix PCR Reaction Mix (Sigma, St. Louis, MO). The negative controls replaced the template DNA with sterile H2O.

The cycles for PCR entailed an initial denaturation at 94°C for 5 minutes, followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 62°C for 30 seconds, and extension at 72°C for 2 minutes. A final extension occurred at 72°C for 5 minutes. All samples were stored at 4°C.

Gel Electrophoresis

To analyze the length of the amplified genetic material, all samples were ran on an agarose gel. A 2.5% Agarose I (Midsci, St. Louis, MO) gel was prepared with 1X TBE buffer (Trise Base 1.1%, Boric acid 5.5%, EDTA 0.2%). The wells were loaded with 15 µl of each sample. A DirectLoad Step Ladder (50bp-3000bp, Sigma, St. Louis, MO) was used as a size standard. The gels ran between 94 and 110 volts. When complete, the gels were stained with an Ethidium Bromide solution on a gentle rocker for 20 minutes, and destained in nanopure water for 10 minutes.

All gels were examines by exposure to UV light for 30 seconds while a camera took a picture. The image was viewed on VersaDoc software on a Mac computer.

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